AD_____

AWARD NUMBER: W81XWH-04-1-0314

TITLE: Prostate Cancer Aggressiveness Genes in Hereditary Prostate Cancer

PRINCIPAL INVESTIGATOR: Kathleen A. Cooney, M.D.
Colin Duckett, Ph.D.

CONTRACTING ORGANIZATION: University of Michigan
Ann Arbor, Michigan 48109-1274

REPORT DATE: March 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
March 2006	Annual	1 Mar 05 – 28 Feb 06

Prostate Cancer Aggressiveness Genes in Hereditary Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0314

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Kathleen A. Cooney, M.D. and Colin Duckett, Ph.D.

E-mail: kcooney@umich.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Michigan
Ann Arbor, Michigan 48109-1274

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

No abstract provided.

15. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)

No keywords provided.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	9	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Introduction: The *EZH2* transcriptional regulator, recently shown by to be overexpressed in prostate cancer specimens that are more likely to recur, maps to 7q35 and represents a candidate gene for inherited prostate cancer susceptibility. This statement is based on the identification of prostate cancer linkage to distal 7q markers in a recently completed genome-wide scan using hereditary prostate cancer families from the University of Michigan Prostate Cancer Genetics Project (PCGP). Relatively little is known about the molecular basis of *EZH2* function or its DNA specificity. The major hypothesis of this proposal is that germline mutations in the *EZH2* gene will predispose to more clinically aggressive forms of prostate cancer and the characterization of these mutations will provide more information about the function of the *EZH2* molecule in prostate cancer and metastasis. To address this hypothesis, the following two specific aims were proposed: 1) to identify germline mutations in *EZH2* that predispose to aggressive prostate cancer in prostate cancer families, and 2) to characterize the functional consequences of *EZH2* mutations specifically focusing on the role of *EZH2* in transcriptional regulation.

Body: To address specific aim 1, we had initially planned to perform a resequencing experiment to identify EZH2 germline variants, particularly single nucleotide polymorphisms (SNPs), that are present at a frequency of 10% or greater and to use these variants to identify the important EZH2 haplotypes. The haplotype-tagging SNPs would be used to determine whether one or more SNPs are associated with either prostate cancer incidence or aggressiveness using the DNA samples form the PCGP. Prior to performing the resequencing, we set out to determine whether there was sufficient published data or information in any of the publicly-available SNP databases that we could utilize to understand the genetic variation in EZH2. Over the past 5 years, these databases (e.g. HAPMAP) have become very complete and have provided population-based data that can be used for research purposes. In the January 2006 version of the International HAPMAP project, we have discovered that 64 SNPs had been typed in ~77 kb of DNA that encompasses EZH2. Of these 64 SNPs, 47 were determined to be polymorphic in the Caucasian sample (CEPH individuals). Using a pairwise tagging strategy, we determined that 12 SNPs (indicated by asterisks in Table below) would be necessary to tag all 47 SNPs at an R^2 level of greater than 0.8. Note that the SNP indicated by rs2302427 is the Asp146His *EZH2* variant described in the revised statement of work. It is the only nonsynonymous variant that has been reported for this gene.

Polymorphic SNPs in EZH2 Gene

	rs2072408	rs3807450	rs3779037	rs10274701	rs4726996
rs887569	rs2072407	rs3757441*	rs1544571	rs2888566*	rs10233740
rs734004	rs6946982*	rs2302427*	rs17171127	rs10952780	rs12704049*
rs734003*	rs3735219*	rs2300150	rs3779038	rs10952781*	rs1880358
rs734002	rs17171118	rs10488070	rs10274535	rs1880355	rs7783459
rs734005	rs17171119	rs6959647	rs9691534	rs10952783*	rs1880357
rs740949	rs6464926	rs3807451*	rs6975291	rs1996996	rs7782553*
rs10268879	rs1061037	rs11764515	rs6943253*	rs12670401	rs2177567

Also in the past year, Bachman et al. [1] reported on an EZH2 mutation screen using DNA samples from 24 men with prostate cancer derived from 10 families that demonstrated evidence of prostate cancer linkage to 7q31-33 markers. In their screen of all 20 *EZH2* exons as well as the 5" and 3' UTRs, only two variants were identified in the open reading frame: Asp146His and a silent nucleotide substitution in exon 15 (1731G>A). Thus these authors concluded that deleterious mutations in *EZH2* likely did not account for the distal

chromosome 7 linkage signal. However, several suggestive associations were seen between specific *EZH2* haplotypes and prostate cancer which supports the plan of using a haplotype-tagging approach for the completion of aim 1.

We plan to genotype of a set of haplotype-tagging SNPs in a set of 925 brothers derived from 361 families in which there is both a brother with prostate cancer and a brother without prostate cancer. As described in Douglas et al.,[2] we will use both family-based association tests as well as conditional logistic regression to determine if any single SNP or haplotype is associated with prostate cancer. The data will then be reanalyzed to determine if any single SNP or haplotype is associated with aggressive prostate cancer. Although we had initially proposed to test the Asp146His *EZH2* (rs2302427) in year one of this grant followed by a haplotype tagging strategy, we were significantly delayed in starting aim 1 due to the regulatory concerns raised by the Office of Research Protections for the Department of Defense Prostate Cancer Research Programs. Thus we have determined that it would be most efficient to perform all of the genotyping and analysis for Aim 1 during the last year of the award.

We have also made significant progress in achieving the goals of Aim 2. We have validated the interaction between EZH2 and REA, primarily by taking an ectopic expression and coimmunoprecipitation approach (Figure 1A and B). In the previous reporting period, we have additionally focued on the development of approaches to examine the nature and biological significance of these interactions. To this end, we begun to analyze the subcellular localization of interaction, using epitope-tagged versions of these proteins (Figure 1B). While the studies are still underway, we have also developed reagents to use biomolecular fluorescence complementation (BiFC) in order to study the interaction between these two proteins in greater detail. Briefly, this approach involves the generation of empirically derived protein chimeras in which the two proteins of interest (in this case EZH2 and REA) are fused to separate moieties of the fluorescent molecule, YFP. Upon fluorescent excitation, YFP emission will only be reconstituted if EZH2 and REA are physically associated, and thus this approach is a significant enhancement over standard coimmunofluorescence. While the imaging studies are currently underway, our preliminary experiments (Figure 2) reveal that, at least by flow cytometry, YFP fluorescence can be readily detected upon coexpression of the appropriate EZH2-REA chimeras.

We have also begun to make inroads in to understanding the functional consequences of the EZH2-REA interaction. While the readouts for EZH2 biological remain complex, we are continuing to develop systems such as chromatin immunoprecipitation to examine the effects of REA and EZH2 on endogenous gene activity, we have also considered the possibility that EZH2 might affect the activity of REA-responsive genes. AS shown in Figure 3, we have found that RNAi-mediated suppression of EZH2 (Figure 3A) can enhance the estradiol-induced expression of the REA-regulated reporter gene, ERE-luc. To date we have observed this effect both in HEK293 cells (Figure 3B) and MDA-MB-231 cells (Figure 3C), and are extending these findings to bother androgen-dependent and –independent prostate cancer lines including PC-3 and LnCaP.

Legend to Figures:

Figure 1: Association between REA and EZH2. (A) Coprecipitation analysis of EZH2 and REA proteins. Expression vectors encoding HA-tagged REA and FLAG-tagged EZH2, or the indicated control plasmids, were transfected into HEK293 cells. Lysates were immunoprecipated with FLAG antibody, and analyzed by immunoblot with the REA-specific HA antibody or a directly conjugated FLAG antibody, as indicated. (C) HEK293 cells were transfeced as in (A) above, except using a glutathione-S-transferase (GST)-tagged EZH2. Lysates were precipitated with glutathione agarose beads and immunoblot for REA as described in (A) above. (C) Coimmunofluorescence analysis of the EZH2-REA interaction.

Figure 2: Preliminary bifluorescence complementation analysis. HEK293 cells were transfected with empty control vectors (upper histogram) or expression vectors encoding REA and EZH2 with different permutations of the amino or carboxy terminal moieties of YFP, and fluorescent reconstitution was evaluated by flow cytometry. As shown in the middle histogram, the combination of the amino half of YFP fused to the carboxy terminal of REA efficiently reconstitutes the remaining half of YFP fused to the carboxy terminal of EZH2.

Figure 3: Effects of EZH2 suppression on REA-regulated transcription. (A) Gen suppressive effects on EZH2 expression following transient transfection of EZH2-specific double-stranded RNAi oligonucleotides into HEK293 cells or MDA-MB-231 cells, as determined by immunoblot. (B) Effects of EZH2 suppression on reporter gene activity of the REA-regulated promoter-luciferase construct, ERE-luc, in HEK293 cells and (C) MDA-MB-231 cells.

Key Research Accomplishments: (new accomplishments in bold)

Specific Aim 1	Annual IRB approval of research protocols. Design and validation of exon-specific primers for EZH2 **Analysis of HAPMAP data outlining the genetic diversity of EZH2** **Planning for genetic analysis of PCGP discordant sibling pairs using haplotype-tagging SNPS**
Specific Aim 2	Establishment of *EZH2*-TAP system Purification and identification of *EZH2*-associated proteins Validation of REA as a physiologically relevant *EZH2*-associated protein Establishment of reporter systems to analyze *EZH2* and REA activity Design and validation of small interfering RNAs (siRNAs) for REA and XIAP **Coimmunofluorescence studies of the EZH2-REA interaction** **Establishment of a bimolecular fluorescence complementation system for dynamic analysis of the EZH2-REA interaction** **Evaluation of the effects of EZH2 and REA in stimulus-responsive gene reporter assays** **Stable suppression of EZH2 and REA levels by lentiviral delivery of short hairpin RNAi's**

Reportable Outcomes: N/A

Conclusions: We have made significant progress toward addressing Specific Aim 1 and have outlined a plan to examine the whether germline genetic variation in EZH2 is associated with prostate cancer using a haplotype-tagging strategy. We have also made major progress toward achieving the goals of Specific Aim 2, particularly in the examination of the functional consequences of the interaction between EZH2 and REA, and additionally we have developed the critical reagents and experimental systems to further address the dynamics and functional significance of the EZH2-REA complex.

References:

1. Bachmann N et al. Mutation screen and association study of EZH2 as a susceptibility gene for aggressive prostate cancer. *Prostate* 2005; **65:**252-259.

2. Douglas JA et al. Identifying susceptibility genes for prostate cancer--a family-based association study of polymorphisms in CYP17, CYP19, CYP11A1, and LH-beta. *Cancer Epidemiol Biomarkers Prev* 2005; **14:**2035-2039.

Appendices: See attached Figures 1, 2, and 3.

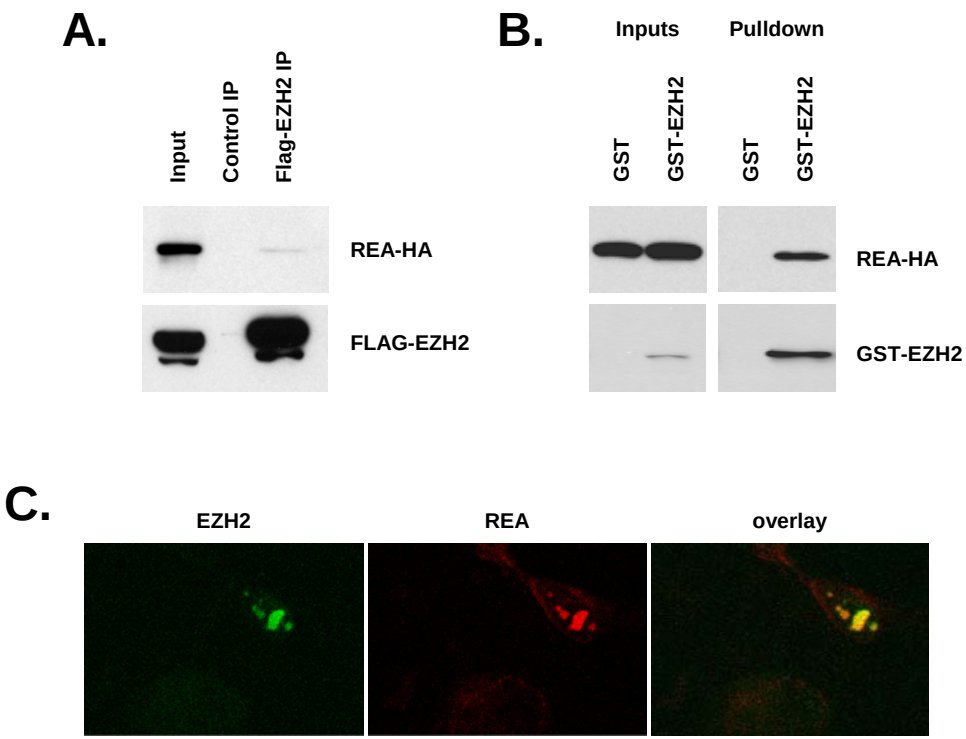
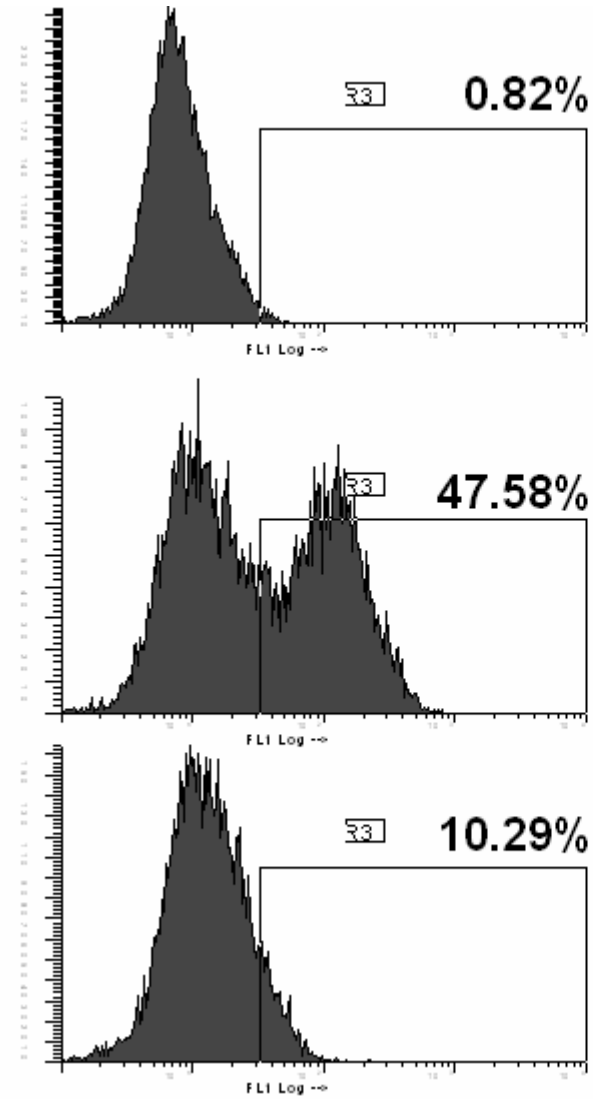

A.
Input
Control IP
Flag-EZH2 IP
REA-HA
FLAG-EZH2
B.
Inputs
Pulldown
GST
GST-EZH2
GST
GST-EZH2
REA-HA
GST-EZH2
C.
EZH2
REA
overlay

Figure 1

Bimolecular fluorescence complementation – FACS analysis



untransfected

REA-YN + EZH2-YC

REA-YC + EZH2-YN

Figure 2

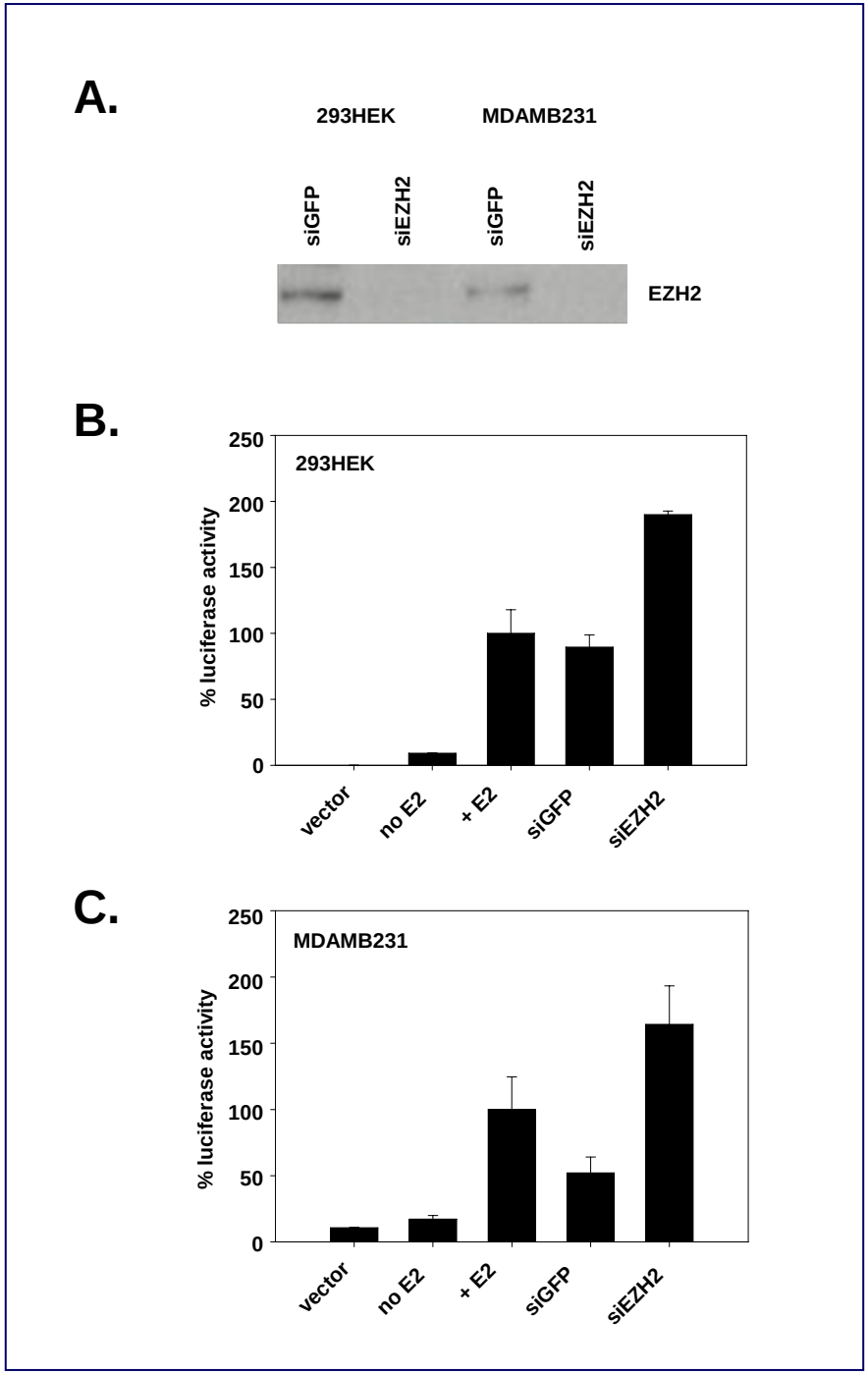

A.
293HEK
MDAMB231
siGFP
siEZH2
siGFP
siEZH2
EZH2
B.
293HEK
% luciferase activity
250
200
150
100
50
0
vector
no E2
+ E2
siGFP
siEZH2
C.
MDAMB231
% luciferase activity
250
200
150
100
50
0
vector
no E2
+ E2
siGFP
siEZH2

Figure 3